Chapter 3

NO_3^- and H^+ fluxes in *Atclcd* mutants of *Arabidopsis thaliana*

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ABSTRACT

In the Arabidopsis genome seven ClC genes have been identified, including AtClCa and AtClCd that are involved in nitrate accumulation in the vacuole and cell expansion, respectively. The effect of NO_3^- on H^+ and Cl^- fluxes from leaf tissue of Arabidopsis was determined for wildtype and and AtClCd T-DNA insertion mutants. When leaf tissue of wildtype plants is exposed to increased levels of nitrate in the external medium the influx of protons and chloride ions is decreased. These effects are absent of much smaller in the AtClCd mutant plants These results are consistent with a function of the AtClCd protein as H^+/anion antiporter.

INTRODUCTION

The protein family of chloride channels and anion transporters (ClC) is widely distributed in prokaryotes and eukaryotes. They play important roles in cell signaling, osmo-regulation, nutrient uptake and distribution, and metabolism. In higher plants the first anion transporter proteins were described in tobacco and their identity was inferred from homology with the ClC family of voltage-gated chloride channels in animals (Lurin *et al.,* 1996). Since then the function of only a few of these proteins in plants has been established. In the Arabidopsis genome seven ClC genes can be identified (*AtClCa-g*), and all of them have been isolated (Hechanberger *et al*., 1996; Geelen *et al.*, 2000; Lv *et al.*, 2009). The intracellular localization of the respective proteins was deduced from expression studies with green fluorescent protein (GFP) fusion proteins (Hechenberger *et al*., 1996; Lv *et al.*, 2009), but direct evidence for their transport activity in plant cells is still lacking. GFP fusion protein studies point to a subcellular localization of AtClCa, -b, -c and -g in the tonoplast, AtClCd and AtClCf in the Golgi membrane and AtClCe in the thylakoid membrane (De Angeli *et al*., 2007; Marmagne *et al*., 2007; Lv *et al*., 2009). However, most of these studies used the 35S promotor, resulting in very high expression levels, possibly resulting in a-typical localization of the fluorescent protein (see Chapter 1). Lv *et al.* (2009) found that the highest expression of AtClCa and AtClCd is in the leaf and the root.
As uptake of Cl^- is normally against its electrochemical gradient, which requires an active mechanism, for instance, through a symporter with H^+ as the second ion (the suggested mechanism in plants (Felle, 1994)). Several mammalian ClCs have been recognized to function as chloride/proton exchangers (Picollo and Pusch 2005; Scheel *et al.*, 2005).

In plants the function and mechanism of the different ClCs is still under debate. A common characteristic of chloride channels is that they are also permeable for nitrate (Pusch *et al*., 1995), a feature that in animals is of limited physiological importance. In contrast, the nitrate transporter function of ClCs in plants could be the most important one. In *Arabidopsis thaliana* AtClCa even appears to be much more selective for NO_3^-, I^- and Br^- than for Cl^- (De Angeli *et al*., 2006). As the concentrations of cytosolic NO_3^- and Cl^- in plant cells are approximately 4 and 10 mM, respectively (Felle, 1994; Miller and Smith, 1996; Lorenzen *et al*., 2004), plant membrane potentials range from -150 to -220 mV (negative inside), and in most soils the concentration of NO_3^- and Cl^- is typically in the low millimolar to micromolar

range (Marschner, 2002), the uptake of nitrate (and of chloride) is only possible by a co-transporter system. Nitrate uptake in roots of *Arabidopsis* is mediated by the well-characterized transporter proteins NTR1 and NTR2. The absorbed nitrate can either be reduced by nitrate reductase, a cytosolic enzyme, or be transported to the shoot. In contrast to the transporters that mediate the uptake into the root, the transporter proteins that are involved in nitrate distribution in various tissues and in the nitrate accumulation in the vacuole are less well studied. Mutant characterization studies indicate that AtClCa (Geelen *et al.*, 2000; De Angeli *et al.*, 2006) and AtClCc (Harada *et al*., 2004) are involved in the regulation of nitrate levels in *Arabidopsis*. Fecht-Bartenbach *et al.* (2007) showed that AtClCd and V-ATPase support growth in expanding cells and they suggest a more complex connections between ClC proteins and the proton gradient. Disruption of AtClCd results in hypersensitivity to concanamycin A, a specific inhibitor of V-type ATPase (Dettmer *et al.*, 2006). De Angeli *et al*., (2006) reported that AtClCa functions as a 2 NO_3^-/1 H^+ antiporter that is able to accumulate nitrate in vacuole. Lv *et al*., (2009), based on sequence comparison with ClCs characterized in other species, postulated that AtClCd, which belongs to the same subclass as AtClCa, may function as an anion/proton antiporter.

In the present study, the effect of nitrate in the experimental solution on Cl^-, H^+ and NO_3^- fluxes in leaf tissue of wildtype and *Atclcd* mutants was monitored with the MIFE technique. The aim was to test the hypothesis that anion transporters located in the tonoplast are essential in cytoplasmic pH homeostasis. Exposure to different anions should not only lead to modifications in the fluxes of other anions, but also to changes in the proton fluxes across the plasma membrane.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (wild type ecotype Columbia and the AtClCd T-DNA insertional mutant) seeds were obtained from the Salk collection (http://signal.Salk.edu/tdna_ protocols.html). For the AtClCd insertional mutant line the SALK line 42895 was selected. Seeds were surface sterilized with gaseous chlorine, sown in pots containing an organic-rich soil (TULIP PROFI No.4; BOGRO B.V. Hardenberg, The Netherlands) and kept in the dark at 4^{o}C for 3 days. The pots were then transferred to a growth chamber with a 16h/8h light/dark cycle and a temperature of 20±2^{o}C for 20 days.

Selection and isolation of the T-DNA insertion mutant of Atclcd

The T-DNA insertion disrupting the *AtClCd* gene was identified in the database at the SALK Institute Genome Analysis Laboratory. Homozygous mutants lines were identified by screening for resistance to kanamycin and by a PCR-based screen with selected primers for the gene and left border primer according to Salk protocol (see Chapter 2). The plant line with the insertion in this gene is referred to as *Atclcd*.

Reverse Transcript PCR analysis

Total RNA was extracted from shoots and purified using the Qiagen RNeasy plant mini kit according to the manufacturer's protocol. RNA was measured by nano drop machine and then first strand cDNAs was synthesized from total RNA (2µg) isolated using reverse transcriptase (Fermentas, USA) and oligo (dT) primer. Tubulin primers were included, for presence of equal amount of cDNA. For amplification, PCR was performed at an annealing temperature of 55^{o}C, using 32 cycles.

MIFE measurements

Net fluxes of Cl^-, NO_3^- and H^+ were measured non-invasively using the MIFE (Micro Electrode Flux Estimation) technique essentially as described in Shabala *et al*., (1997), Newman (2001) and Lanfermeijer *et al.* (2008). Briefly, microelectrodes were pulled and then dried in an oven at 200^{o}C overnight. To improve the stability of the liquid ion exchange cocktails (LIX) the electrodes were coated with a hydrophobic material (tributylchlorosilane 90796; Sigma-Aldrich, Milwaukee) for 10 min in the

same oven under a steel cover. Then the cover was removed and the electrodes were left to dry at 200°C for another 20 min.

The electrodes were back-filled with 0.5 M KCl in the case of Cl^--specific electrodes, 15 mM NaCl + 40 mM KH_2PO_4 in the case of H^+-specific electrodes and 0.5 M KNO_3 + 0.1 M KCl in the case of NO_3^--specific electrodes. All the back-fill solutions were adjusted to pH 6 with NaOH. Immediately after back-filling, the electrode tips were front-filled either with a commercially available LIX, ionophore 24902 for Cl^- (Cl^- -specific electrodes were used after an overnight 'maturation' period) and 95297 for H^+ (Fluka; Busch, Switzerland) or with a LIX consisting of 0.5% methyltridodecylammonium nitrate (MTDDA), 0.084% methyl-triphenyl-phosphonium bromide (MTPPB) and 99.4% n-phenyloctyl ether (NPOE) for the NO_3^- -specific electrodes (Table 1).

Ion-specific Electrode	Electrode tip fill	Backfill	Specificity[a]	Response[b] (mV/decade)
H^+	Ionophore 95297	15 mM NaCl, 40 mM KH_2PO_4	H^+/Li^+=108	51
Cl^-	Ionophore 24902	0.5 M KCl	Cl^-/I^-=10	48
NO_3^-	0.5% MTDDA NO_3^-[c],0.084% MTPPB[d] and 99.4% NPOE[e]	0.5 M KNO_3, 0.1 M KCl	Cl^-/I^-=8	56

Table 1: Composition of the ion-specific electrodes and their characteristics as used in the MIFE.experiments. [a]: specificity ration with the ion which interferes the most with the studied ion. [b]:The response value is the change in the measured potential when the pIon (e.g. pH, pCl or pNO_3) changes 1 unit.[c]: methyltridodecylammonium nitrate; [d]: methyl-triphenyl-phosphoni bromide; [e]: n-phenyloctyl ether

The epidermis was removed from the abaxial side of the leaf. Leaf material was immobilized on a glass capillary using grease with the abaxial side exposed to the solution and was placed in a measuring chamber with a transparent bottom. The chamber was filled with 1 ml of the basic measuring solution (1 mM KCl, 0.5 mM $CaCl_2$, pH 5.8 for H^+ and Cl^- measurements or 0.1 mM NH_4NO_3, 0.2 mM $CaSO_4$, pH 5.8 for NO_3^- measurements), submerging the leaf material. The whole chamber was placed on a Nikon TMS inverted microscope. The ion-selective microelectrodes were mounted at an angle between 30° and 40° with the horizontal in a holder (MMT-

5Narishige) on a micromanipulator (PCT; Luigs and Neumann) that was driven by a computer-controlled motor (MO61-CE08; superior Electric). All electrodes were calibrated before and after use in a series of solutions with concentrations in the expected range of the ions in the experimental solutions. The medium in the chamber was continuously replaced using a flow-through system (with a flow rate of approximately 3 ml/min). A system of taps allowed changes of the medium from outside the Faraday cage, which enclosed the whole set-up. Net fluxes of Cl^-, NO_3^- and H^+ were recorded in response to exposure of the leaf material to solutions of different solute composition (Table 2).

Measurement	Conditions applied				
	NaCl	CholinCl	Mannitol	KNO_3	KCl
H^+ Fluxes	+	+	+	+	+
Cl^- Fluxes	+	+	+	+	
$NO3^-$ Fluxes				+	+

Table 2. Osmotic conditions tested on *Arabidopsis* wild type and mutant plants for the indicated flux measurements. "+" indicated monitored

RESULTS

Isolation of a homozygous knockout line and gene expression

We obtained one T-DNA insertion line in the Colombia ecotype background from the Salk collection under number SALK-42895. The exact location of the T-DNA insertion for the locus At5g26240 was obtained from the TAIR database (http://www.arabidopsis.org). As shown in figure 1, the T-DNA insertion in Salk-42895 was located in the fourth intron of the gene. The location of the T-DNA in the gene was confirmed by PCR according to the protocol of the SALK consortium. To study *AtClCd* gene expression, RT-PCR was performed using gene specific primers. Figure 1 shows the expression of *AtClCd*, using tubulin as an internal standard. The RT-PCR products confirmed that high expression levels of AtClCd are present in the shoot. RT-PCR also confirmed that in homozygous mutant plants the full transcript of *AtClCd* is absent (Figure 1). This suggests that the T-DNA insertion results in a null allele. Our results confirm an earlier study that showed high expression levels of *AtClCd* in root and shoot (Lv *et al*., 2009).

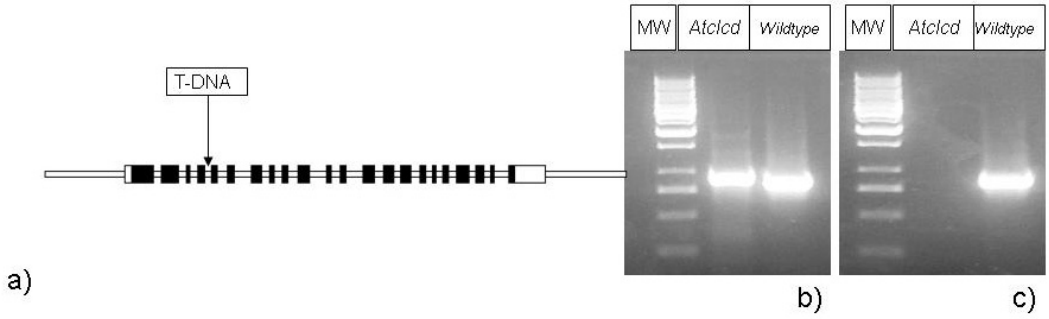

Figure 1. The T-DNA insertion in the *AtClCd* gene **a**: A schematic representation of the position of the T-DNA insertion in *AtClCd* gene. **b**: Tubulin primer experiment showing presence of equal amount of cDNA. **c**: The absence of expression in *AtClCd* gene in its T-DNA insertion lines. The wildtype and the Atclcd genotype were analysed using the primers shown in table 1 of Chapter 1. The *Tubulin* transcript levels are shown as a loading control.

KNO_3–induced proton fluxes are different in wildtype and Atclcd mutant plants

Based on the similarity of AtClCa and AtClCd we hypothesize that AtClCd is also located in the tonoplast, functions as a H^+/NO_3^- antiporter and is involved in nitrate accumulation in the vacuole. Based on these characteristics we predicted that increasing the extracellular nitrate concentration would lead to distinct differences between the *Atclcd* mutants and wildtype plants: 1. Since in the mutant excess nitrate

cannot be stored efficiently in the vacuole, the cytosolic pH will increase more in the mutants compared to wildtype. In wildtype plants the cytosolic pH will be kept lower due to the exchange of nitrate from the cytosol for protons from the vacuole. Furthermore, the reduction of nitrate in the cytosol will consume one proton per NO_3^- reduced to nitrite and several more when reduced further to ammonia. 2. The influx of nitrate will affect the chloride influx in the mutants more than in wildtype plants as accumulation of nitrate in the cytoplasm will likely reduce further uptake of anions. 3. The increase in the nitrate influx is expected to be transient in both genotypes, but will be more pronounced in wildtype plants since the capacity to store nitrate is higher. Addition of nitrate (400 μM and 6 mM KNO_3) resulted in an immediate increase in the influx of nitrate in the leaf tissue (Figure 2). The size of the transient increase of the nitrate influx did not differ significantly between wild type and AtClCd mutant plants.

Increasing the nitrate concentration in the external medium resulted in a reduction of the proton influx in wildtype plants (Figure 3). In *Atclcd* mutant plants nitrate increased the proton influx even further. When DCCD was added to the medium the influx of protons was dramatically reduced, while in wildtype plants the influx remained at the same low level.

Effect of external KNO_3 on chloride flux

In order to check the effects of increasing the external concentration of nitrate on chloride fluxes, we measured Cl^- fluxes before and after nitrate treatment. AtClCa has been shown to be selective for both NO_3^- and Cl^-. Furthermore, NO_3^- generally suppresses Cl^- fluxes and accumulation (Bar *et al.*, 1997; Kafkafi *et al*., 1982; Adler and Wilcox, 1995) and decreases the Cl^- influx, in particular the flux into the vacuole (Britto *et al*., 2004). As shown in figure 4 250 μM KNO_3 indeed decreased the chloride influx and induced an efflux. In contrast, in *Atclcd* mutant plants, addition of KNO_3 only has a transient effect on chloride flux and concentration, as after approximately 1.5 minute the flux returns to pre-addition values.

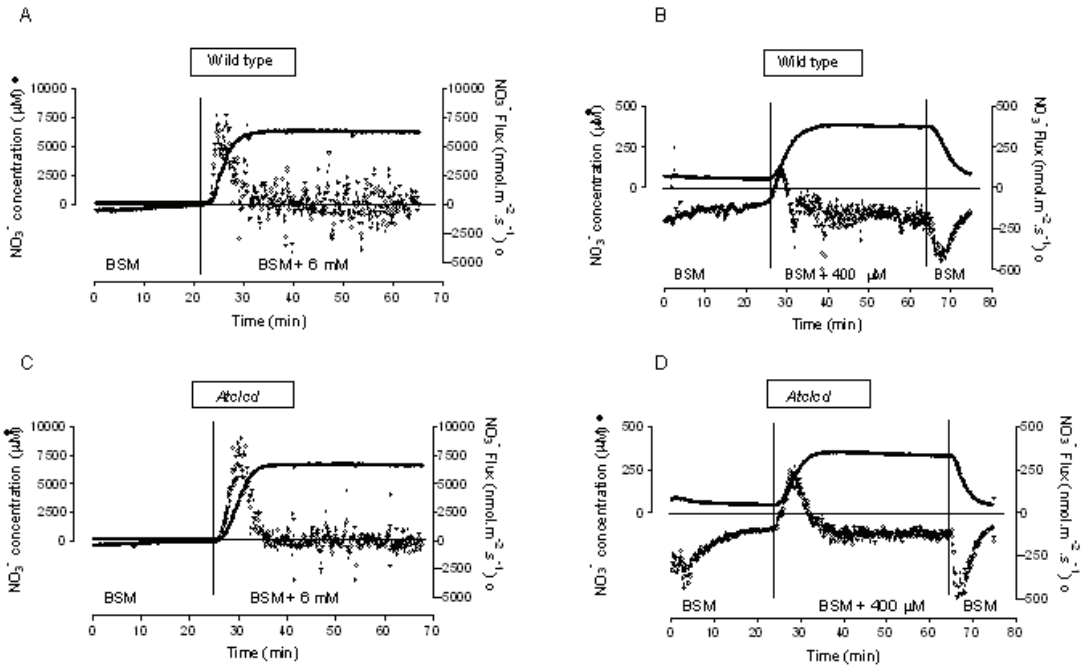

Figure 2. Typical changes in nitrate fluxes from wildtype *Arabidopsis thaliana* (panels A and B) and the *Atclcd* genotype (panels C and D) leaf tissue induced by 6 mM (panels A and C) or 400 µM of KNO_3 (panels B and D). The vertical line indicates the moment when the basal salt medium was slowly replaced by BSM with the supplement. When the flux becomes more positive, this means either an increase of the influx or a reduction of the efflux. Typical experiments of at least three experiments are shown.

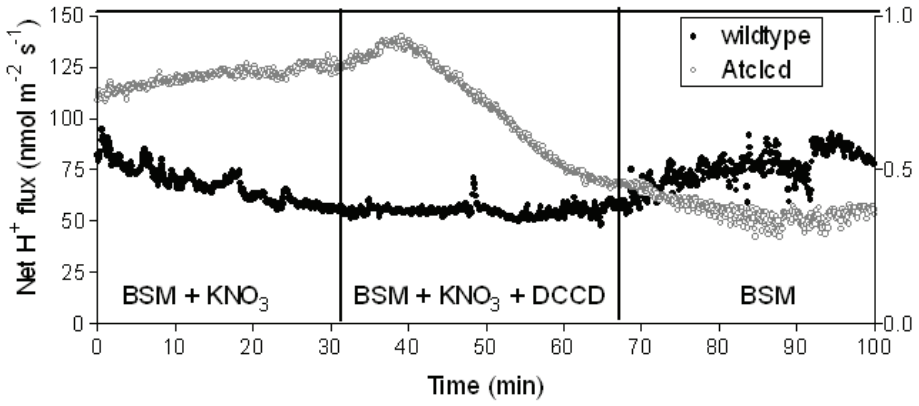

Figure 3. The effect of DCCD on nitrate induced proton fluxes from wildtype *Arabidopsis thaliana* and the *Atclcd* genotype leaf . The vertical lines indicate the moments when the medium was slowly replaced by the next medium as indicated. The concentations used were 50 mM KNO_3 and 20 µM DCCD. Typical experiments of at least three experiments are shown.

DISCUSSION

Relation between NO_3^- and H^+ fluxes in Atclcd mutant plants

The physiological characterization of *Arabidopsis* mutants suggested the involvement of AtClCa (Geelen *et al*., 2000; De Angeli *et al*., 2006) and AtClCc (Harada *et al*., 2004) in the regulation of nitrate levels in plants. The bacterial ClC-ec1 protein (Accardi and Miller, 2004) and the human ClC4 and ClC5 proteins (Picollo and Push, 2005; Scheel *et al.*, 2005) that are located in the membranes of intracellular vesicles, have been shown to function as proton/chloride exchangers, rather than passive chloride channels. In plant cells AtClCa functions as a 2 NO_3^-/1 H^+ antiporter facilitating the accumulation of nitrate in the vacuole (De Angeli *et al.*, 2006), with a selectivity sequence of $NO_3^- = I^- > Cl^-$. AtClCd has been shown to co-localize in the trans-Golgi network with VHA-a1, a subunit of the proton transporting V-Type ATPase (Fecht-Bartenbach *et al*., 2007).

In plant cells, the plasma membrane H^+-pumping ATPase is the primary active transport system and responsible for generating the membrane potential (Assman and Haubrick, 1996). Transport of a cation in the opposite direction or an anion in the same direction is required prevent extreme hyper-polarization of the membrane and allow the build up a steep proton gradient. Therefore different factors like activation of anion channels or changes in pH of the medium may lead to changes the membrane potential and the cytosolic pH. Also plasma membrane anion channels play a central role in the regulation of the cytosolic pH of plant cells (Johannes *et al.*, 1998). The results of our experiments show that application of KNO_3 leads to an H^+ efflux (seen as a reduction of the influx). This efflux is, as is evident from its sensitivity to the ATPase inhibitor DCCD, carried by the plasma membrane H^+-ATPase. This NO_3^--induced H^+ efflux confirms reports by Garnet *et al.* (2003) on eucalypt and Segonzac *et al.* (2007) on *Arabidopsis*. In mutant plants the addition of KNO_3 has much less of an impact on the H^+ fluxes, demonstrating that the presence of an H^+/anion antiporter is essential for the nitrate-induced effects on the plasma membrane proton pump.

Interactions between uptake of Cl^- and NO_3^- ions in Atclcd mutant plants

Under natural condition, the presence of nitrate in the soil can reduce the toxic effect of excess Cl^- (Bar *et al*.,1997). Nitrate can reduce the influx Cl^- in plant cells

and Cl^- accumulation in plant tissue (Glass and Siddiqi, 1985; Adler and Wilcox, 1995). In our experiments the addition of NO_3^- led to an increase in the Cl^- efflux (decreased Cl^- influx) and to a higher external Cl^- concentration when wildtype plants were studied. In the *Atclcd* mutant plants addition of nitrate resulted in a short, transient change in the chloride flux, but not in a sustained efflux (Figure 4). These results are consistent with a function for AtClCd as an H^+/anion antiporter that can transport both nitrate and chloride across the tonoplast.

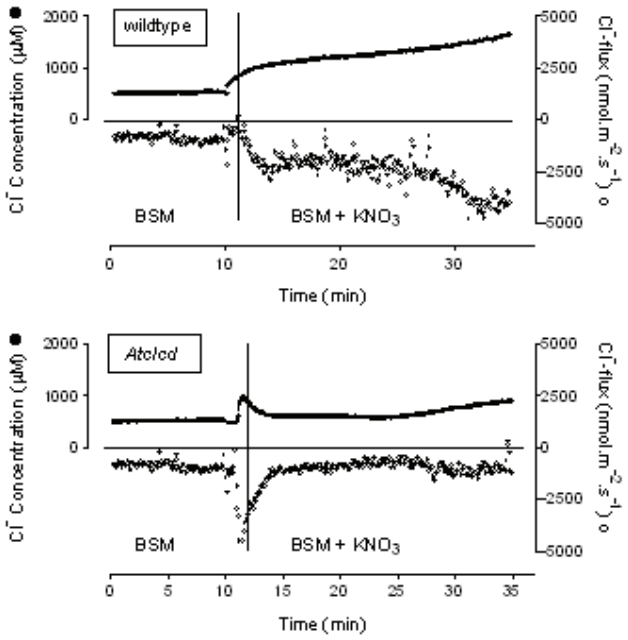

Figure 4. Typical changes in chloride fluxes from wildtype *Arabidopsis thaliana* and the *Atclcd* genotype leaf tissue induced by 250 µM KNO_3. The vertical line indicates the moment when the basal salt medium was slowly replaced by BSM with the supplement. Typical experiments of at least three experiments are shown.

Although the selectivity of the bacterial and the *Arabidopsis* ClC transporters is clearly different, (in *Arabidopsis* NO_3^-, I^- and $Br^- > Cl^-$ (De Angeli *et al.*, 2006), in bacteria $Cl^- >$ Br-, NO_3^- and SO_4^- (Accardi and Miller, 2004)), it is clear that in both NO_3^- and Cl^- could enter the cells via the ClC transporters. Furthermore, addition of NO_3^- decreases the Cl^- influx into the vacuole (Britto *et al*., 2004). These and our data suggest that in plant cells the accumulation of chloride and nitrate in the vacuole is based on competition for transporter activity and that the transporter that mediates this transport might be the AtClCd protein.

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